Inhibition of Human DNA Topoisomerase II by Hydroquinone and *p*-Benzoquinone, Reactive Metabolites of Benzene

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Chronic exposure of humans to benzene (BZ) causes acute myeloid leukemia (AML). Both BZ and therapy-related secondary AML are characterized by chromosomal translocations that may occur by inappropriate recombinational events. DNA topoisomerase II (topo II) is an essential sulfhydryl (SH)-dependent endonuclease required for replication, recombination, chromosome segregation, and chromosome structure. Topo II cleaves DNA at purine(R)/pyrimidine(Y) repeat sequences that have been shown to be highly recombinogenic *in vivo*. Certain antineoplastic drugs stabilize topo II–DNA cleavage complexes at RY repeat sequences, which leads to translocations of the type observed in leukemia. Hydroquinone (HQ) is metabolized to *p*-benzoquinone (BQ) in a peroxidase-mediated reaction in myeloid progenitor cells. BQ interacts with SH groups of SH-dependent enzymes. Consequently, the aims of this research were to determine whether HQ and BQ are topo II inhibitors. The ability of the compounds to inhibit the activity of topo II was tested using an assay system that depends on the conversion, by homogeneous human topo II, of catenated kinetoplast DNA into open and/or nicked open circular DNA that can be separated from the catenated DNA by electrophoresis in a 1% agarose–ethidium bromide gel. We provide preliminary data that indicate that both HQ and BQ cause a time and concentration (μM)-dependent inhibition of topo II activity. These compounds, which potentially can form adducts with DNA, have no effect on the migration of the supercoiled and open circular forms in the electrophoretic gradient, and BQ-adducted KDNA can be decatenated by topo II. Using a pRYG plasmid DNA with a single RY repeat as a cleavage site, it was determined that BQ does not stimulate the production of linear DNA indicative of an inhibition of topo II religation of strand breaks by stabilization of the covalent topo II–DNA cleavage complex. Rather, BQ most probably inhibits the SH-dependent topo II by binding to an essential SH group. The inhibition of topo II by BQ has implications for the formation of deleterious translocations that may be involved in BZ-induced initiation of leukemogenesis. — Environ Health Perspect 104(Suppl 6):1265–1269 (1996)

Key words: benzene, hydroquinone, *p*-benzoquinone, topoisomerase II, translocations

Introduction

Benzene (BZ), a widely used industrial chemical and ubiquitous environmental pollutant, is a Class I carcinogen that causes secondary acute myelogenous leukemia (AML) in humans who are chronically exposed (*1–4*). Benzene hematotoxicity occurs when its hepatic metabolites (*5,6*), such as phenol, catechol and hydroquinone (HQ) are transported to the bone marrow (*7,8*) and further oxidized in a peroxidase-mediated (*9–11*) reaction to biologically reactive intermediates such as *p*-benzoquinone (BQ), which can interact with the genome and potentially affect hematopoiesis.

Because of the association between BZ exposure and an increased incidence of AML, it is important to determine whether BZ can cause genotoxic effects of the types observed in secondary AML (*4*). Cytogenetic studies of the karyotypes of workers occupationally exposed to BZ have demonstrated the presence of nonrandom chromosomal aberrations such as breaks, gaps, and—infrequently—rearrangements on chromosomes 2, 4, and 7 (*12,13*). In another study (*14*), an individual whose exposure to BZ was considered to be high, showed a reciprocal familial chromosomal translocation [t(3;16)(q11;q11)] and karyotype abnormalities in 100% of the marrow cells, which included t(9;10) and t(4;15) translocations. A t(4;11) (q21;q23) has been reported in a furniture worker who had a "benzene intoxication" for 3 months prior to the development of acute lymphoblastic leukemia (ALL) (*15*). This translocation has also been reported (*16*) in an individual with therapy-related myelodysplasia (preleukemia).

Topo II, a sulfhydryl (SH)-dependent endonuclease essential for replication, recombination, chromosome segregation, and chromosome structure (*17*), catalyzes the relaxation of supercoiled DNA by the transient cleavage and religation of both strands of duplex DNA (*17*). It cleaves DNA at purine(R)/pyrimidine(Y) repeat sequences (*18*) that have been shown to be highly recombinogenic in eukaryotes *in vivo* (*19,20*). There appears to be sequence homology between topo II cleavage sites and the sequences at the t(4;11) and t(9;11) translocation breakpoint junctions, suggesting that topo II may function in chromosomal translocations at chromosomal band 11q23.The epipodophyllotoxin class of antineoplastic drugs enhances topo II-mediated chromosomal breakage by stabilizing the topo II–DNA cleavage complex and thus decreasing the religation of the nicked DNA strands (*17*) and resulting in chromosomal changes at the cellular level of the types observed in leukemia. The nonrandom association of 11q23 chromosomal translocations with epipodophylotoxin chemotherapy suggests that the 11q23 translocation breakpoints within the *MLL* gene may coincide with cleavage sites for topo II. Epipodophyllotoxin-induced *in vitro* topo II cleavage sites do, in fact, correspond with chromosome 11q23 translocation breakpoints (C Felix and M-A Bjornsti, personal communication).

BQ, in common with the antineoplastic drugs, is an alkylating agent that has a high

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Abbreviations used: AML, acute myelogenous leukemia; BQ, *p*-benzoquinone; BZ, benzene; dKDNA, decatenated kinetoplast DNA; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; HQ, hydroquinone; KDNA, kinetoplast DNA; lKDNA, linear kinetoplast DNA; topoII, topoisomerase II.

propensity to react with SH groups of proteins and has been shown to inactivate several SH-dependent proteins (*21–24*). The fact that these quinones also induce chromosomal aberrations suggests that they might be affecting chromosomal translocations by inhibition of topo II.

We report preliminary results that indicate that both HQ and BQ, at micromolar concentrations, cause the concentration-dependent inhibition of topo II activity, probably by interaction with an essential SH group of topo II rather than by stabilization of the topo II–DNA cleavage–religation complex.

Materials and Methods

Materials

Ehtylenediaminetetraacetate (EDTA), chloroform, isoamyl alcohol, HQ, and BQ, were obtained from Fisher Scientific Co. (Pittsburgh, PA). Electrophoresis grade agarose was purchased from Bethesda Research Laboratories (Bethesda, MD), and ethidium bromide (EtBr), Sarkosyl, bromphenol blue, and glycerol were obtained from the Sigma Chemical Co. (St Louis, MO). Boehringer Mannheim (Indianapolis, IN) was the source of proteinase K. Homogeneous human placental topo II (170 kDa) was obtained from TopoGen, Inc. (Columbus, OH). The enzyme was free of topo I activity and nuclease contamination. A unit of topo II activtiy is that amount of enzyme that decatenates 0.2 µg of catenated kinetoplast DNA (KDNA) in 15 min at 37°C. KDNA, catenated mitochondrial DNA of *Crithidia fasciculata,* decatenated KDNA (dKDNA), linear KDNA (lKDNA), supercoiled pRYG DNA, linear pRYG DNA (lpRYG DNA), 10× assay, cleavage and gel-loading buffers and 5× stop buffer, and sodium dodecyl sulfate (SDS) were also obtained from TopoGen, Inc. All other reagents were of the highest grade available.

Methods

Topo II Assay. The activity of topo II was tested using an assay system that depends on the conversion by topo II of highly catenated KDNA into open and/or nicked open circular DNAs. KDNA is an aggregate of interlocked DNA minicircles of 2.5 kb that form a large network of high molecular weight DNA that does not penetrate an agarose gel matrix. Incubation of KDNA with topo II results in decatenation of KDNA with the release of minicircular decatenated KDNAs that electrophoretically migrate rapidly into an agarose gel.

The standard reaction contained 0.2 µg KDNA and 2 u of topo II in 50 mM Tris–HCl buffer, pH 8.0, containing 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT in a final volume of 20 µl. Incubation was carried out at 37°C for 15 min and terminated by the addition of 4 µl of stop/loading buffer consisting of 5% Sarkosyl, 0.0025% bromphenol blue, 25% glycerol. Marker DNAs were run to confirm the type of decatenated product produced in the reaction. Fifteen microliters of mixture generally containing 0.15 µg DNA was loaded into a well of a 1% agarose gel containing 0.16 µg/ml EtBr in 1× Tris–acetate (TAE) buffer. Gels were run at room temperature at a constant voltage of 50 mV for 4 hr. The 1% agarose gel has a range of separation of linear DNA molecules of 0.5 to 7 kb. The gels were dried and the positions of the various species of DNA in the gel were localized, in comparison with DNA markers, by exposure of the EtBr-intercalated DNA to ultraviolet light.

Analysis of the ability of HQ and BQ to inhibit topo II activity was carried out using the same assay except that the compounds were preincubated for 15 min with topo II at concentrations listed in the legend to Figure 1. The concentrations of the metabolites stated were in excess of the essential concentration of DTT in the enzyme preparation.

Assay to Determine Whether HQ and BQ Inhibit Topo II by Stabilization of the Topo II-mediated DNA Cleavage–Religation Complex

Eucaryotic topo II preferentially cleaves alternating purine/pyrimidine repeat sequences in DNA (*18*). A plasmid construct (pRYG) consisting of pUC19 plasmid containing a 245-bp fragment of the human β-globin gene promoter with a 54-bp purine (R)/pyrimidine (Y) repeat sequence that serves as a high-affinity topo II recognition and cleavage site was used as a substrate. In a cleavage assay, pRYG allows the detection of two kinds of topo II inhibitors: those that stimulate formation of cleavable complexes such as the antineoplastic drugs teneposide (VM-26) and mAMSA, and agents that antagonize topo II action on the DNA (i.e., may interact with the active site of the enzyme). The cleavage assay is based on the production of a linear pRYG cleavage product in the presence of agents that stabilize the covalent topo II–DNA cleavage complex by inhibiting the topo II-mediated religation reaction.

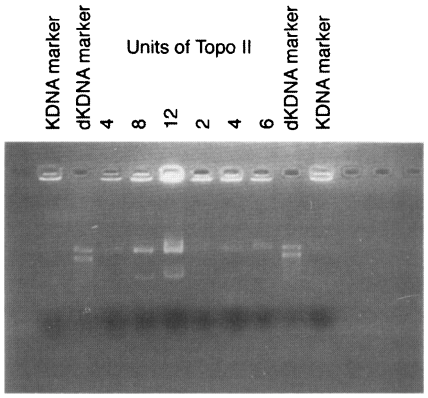

Figure 1. The decatenation of catenated kinetoplast DNA as a function of topo II units. KDNA (0.2 µg) was incubated for 30 min at 37°C with varying units of human placental topo II in 50 mM Tris–HCl buffer, pH 8.0, containing 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT in a final volume of 20 µl. The reaction was terminated by the addition of 4 µl of stop/loading buffer consisting of 5% Sarkosyl, 0.0025% bromphenol blue, and 25% glycerol. A 15-µl sample (0.15 µg DNA) was loaded into each well of a 1% agarose/2 µg/ml ethidium bromide gradient. The gel was run at room temperature at a constant voltage for a length of time (generally 4 hr) sufficient to allow the migration of the smallest DNAs to traverse 80% of the length of the gel. Bands were visualized by exposure of the gel to UV light and photographed. KDNA, catenated kinetoplast DNA marker; dKDNA, decatenated KDNA marker.

In the cleavage reaction, pRYG plasmid (0.25 µg) was incubated with 3 to 6 units of topo II in 30 mM Tris–HCl buffer, pH 7.6, containing 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$, 60 mM NaCl in a final volume of 20 µl. BQ was added at a concentration of 20 to 200 µM over the concentration of mercaptoethanol in the reaction. Incubation was carried out at 37°C for 30 min. The reaction was terminated by the addition of 2 µl of 10% SDS. The mixture was digested with proteinase K (50 µg/ml) for 15 min and extracted with an equal volume of chloroform: isoamyl alcohol (24:1). The upper phase (20 µl) was loaded into a well of a 1% agarose gel containing 2 µg/ml EtBr.

Results

Decatenation of KDNA by Topo II

Incubation of topo II (2–12 units) with KDNA resulted in a concentration-dependent decatenation of KDNA into open and

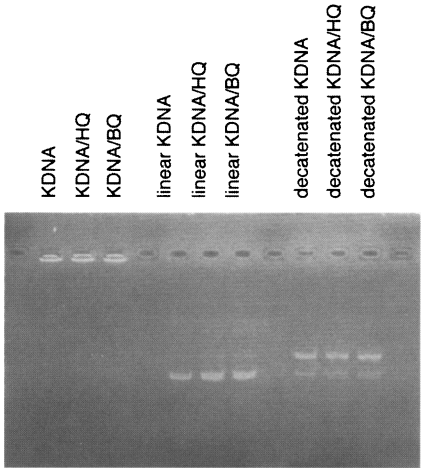

Figure 2. The effect of HQ and BQ on the electrophoretic mobility of the various species of KDNA produced in the topo II decatenation assay. KDNA, dKDNA, and IKDNA marker DNAs (0.2 µg) were reacted with 6 µM HQ or BQ and subjected to gel electrophoresis as described in the caption to Figure 1.

Figure 3. Decatenation of BQ-adducted KDNA by topoisomerase II. KDNA (75 µg) was incubated for 1 hr at 37°C with 6 µM BQ to form KDNA adducts. The unreacted BQ was removed by treatment with excess 2-mercaptoethanol and the BQ–mercaptoethanol separated from the BQ-adducted KDNA by gel exclusion chromatography. Both DNAs (0.2 µg/well) were added to the wells of a 1% agarose/ethidium bromide gel and electrophoresed as described in the caption to Figure 1.

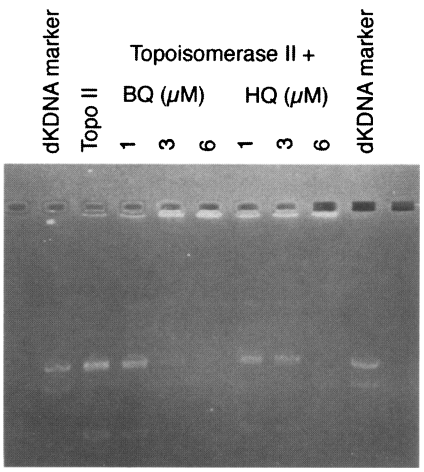

Figure 4. Inhibition of topo II activity by HQ and BQ. Topo II (2 U) was preincubated with the stated concentrations of HQ or BQ for 15 min at 37°C before the addition of KDNA (0.2 µg), after which the reaction was allowed to proceed for 30 min. The gel was loaded and the electrophoresis of the products of the reaction was carried out as described in the caption to Figure 1. Abbreviations: KDNA catenated kinetoplast DNA, dKDNA decatenated kDNA.

nicked open circular DNA (Figure 1). The amount of decatenation that occurred was a function of time and temperature (data not presented). Thirty minutes at 37°C were found to be optimal. This experiment is representative of four experiments that gave identical results.

Effect of Hydroquinone and *p*-Benzoquinone on the Ability of Topo II to Decatenate KDNA

It is well known that HQ and BQ form covalent adducts with DNA (*25*). Therefore, it was necessary to ascertain whether the formation of adducts with KDNA during the course of the reaction had any effect on the migration in the electrophoretic gradient of any of the DNA species produced. The gel profile presented in Figure 2 shows that incubation of the various marker DNAs with HQ or BQ at the highest concentration (6 µM) of metabolite used in the assay for inhibition of topo II decatenation of KDNA had no effect on the electrophoretic migration of the DNA marker species in the gel. To determine whether BQ-adducted DNA interferred with the ability of topo II to decatenate KDNA, KDNA was reacted with 6 µM BQ for 1 hr at 37°C (topo II assay conditions) to allow KDNA to form any possible adducts with BQ. The unreacted BQ was removed and the BQ-adducted KDNA was used, along with KDNA, as substrate in the topo II decatenation assay. As can be seen in Figure 3, the presence of BQ-adducted KDNA did not interfere with the cleavage and religation reactions of topo II on the basis that the same DNA species were produced from both BQ-adducted KDNA and KDNA. Topo II is very sensitive to salt concentrations; consequently, HQ and BQ were prepared and/or diluted in deionized water to maintain the optimal salt concentration in the reaction.

The ability of HQ and BQ to inhibit the decatenation of KDNA by topo II was tested. Preincubation of topo II with HQ or BQ for 15 min over a concentration range of 1 to 6 µM prior to incubation with KDNA caused a concentration-dependent inhibition of topo II decatenation of KDNA. BQ inhibited at a concentration of 3 µM or higher, as indicated by the lack of decatenated and/or open or nicked open circular forms of DNA (Figure 4). HQ did not show inhibition of topo II at 3 µM but showed a complete inhibition at 6 µM. The experiment presented is representative of three similar experiments.

Effect of Hydroquinone and *p*-Benzoquinone on the Topo II-mediated DNA Cleavage–Religation Reaction

Several potent and clinically relevant antineoplastic agents known to cause secondary leukemia stabilize the topo II-DNA cleavage complex by inhibiting the topo II-mediated religation reaction (*17,26*). When this stabilization occurs, the DNA fragments resulting from the double-strand breaks appear in the gel as a linear species. As can be seen in Figure 5, when topo II was incubated in the cleavage assay with pRYG plasmid, which contains a single RY topo II cleavage site, in the presence of 200 µM BQ, no linear pRYG DNA was produced. BQ, at 60 times the concentration that inhibits topo II activity in the decatenation

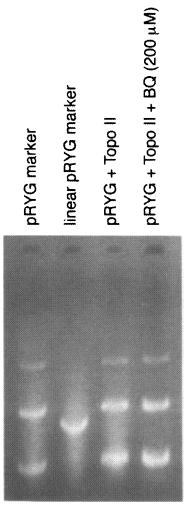

Figure 5. The effect of BQ on the topo II-mediated cleavage–religation complex. pRYG plasmid (0.25 µg) containing a high-affintiy topo II purine–pyrimidine repeat sequence was incubated with 6 U of topo II in cleavage buffer consisting of 30 mM Tris-HCl, pH 7.6, 3 mM ATP, 15 mM 2-mercaptoethanol, 8 mM $MgCl_2$, and 60 mM NaCl. Because of the 15 mM 2-mercaptoethanol in the reaction to protect the SH of topo II, it was necessary to add excess BQ over the amount of mercaptoethanol that will react with the BQ. Consequently, BQ was added at a concentration of 200 µM over the concentration of 2-mercaptoethanol in the reaction. The reaction was carried out for 30 min at 37°C and was terminated by the addition of 2 µl of 10% SDS. The mixture was digested with proteinase K and extracted with chloroform:isoamyl alcohol (24:1). A sample of the upper phase was loaded onto a 1% agarose/EtBr gel and electophoresed.

assay, appears unable to stabilize the topo II-mediated DNA cleavage–religation complex. Similar results were obtained at concentrations of BQ between 20 and 200 μM. Although not shown in this gel, linear DNA was produced from pRYG DNA by topo II when the incubation was carried out in the presence of 200 μM mAMSA, an agent that stabilizes the cleavage complex. This experiment was carried out 4 times with identical results.

Discussion

Topo II is involved in many fundamental processes occurring on the chromosome, including recombination events (*27*). Central to the physiological function of topo II is its ability to introduce and religate site-specific double-stranded breaks in the genome. Topo II serves as a therapeutic target for various antibacterial, antiparasitic antifungal, antiviral, and antineoplastic drugs (*26*). These therapeutic drugs interfere with the cleavage–religation reaction of topo II by stabilizing the cleaved state as an enzyme–DNA–drug ternary cleavable complex. Topo II is a SH-dependent endonuclease and as such is inhibited by agents that form covalent adducts with SH groups. Both BZ-induced and therapy-related AML are characterized by chromosomal translocations or deletions that may occur by inappropriate recombinational events and may cause the conversion of a protooncogene to an oncogene or the loss of a suppressor gene resulting in the initiation of leukemogenesis. These events may occur by inhibition of the activity of topo II via an effect on an essential SH group or by the stabilization of the topo II–DNA cleavage complex.

HQ is converted in a peroxidase-mediated reaction in the myeloblast to BQ that covalently binds to SH-dependent proteins and inhibits their activity. Consequently, it was important to ascertain whether HQ or BQ could affect the activity of topo II and thus play a possible role in the initiation of BZ-induced AML. BQ was found to inhibit, in a concentration-dependent manner, the decatenation of highly catenated kinetoplast DNA by human placental topo II (Figure 4), whereas HQ only showed inhibition at the highest level tested, 6 μM. The ability of the compounds to form adducts with the KDNA substrate had no effect either on the migration of the decatenated supercoiled and open circular products in the electrophoretic gradient (Figure 2) or the ability of topo II to use BQ-adducted DNA as a substrate (Figure 3).

Using a pRYG plasmid with a 54 bp RY repeat, for which topo II has a very high affinity, it was determined that BQ, at a concentration 50-fold higher than the concentration that inhibits the decatenating activity of topo II, did not stimulate the formation of linear pRYG DNA (Figure 5) indicative of an inhibition of a topo II religation of double-strand breaks by stabilization of the covalent topo II–DNA cleavage complex. Rather, BQ appears to interact directly with the SH-dependent topo II presumably at an essential SH group at the active site of the enzyme. Although these experiments were carried out in vitro, it is likely that topo II is accessable to HQ and BQ *in vivo* because topo II is present in high concentration in chromatin as a protein scaffold (*28–30*) and because both HQ and BQ form adducts with nuclear DNA in myeloid cells (*31,32*). The inhibition of topo II activity by these bioreactive metabolites of BZ may have implications for the formation of deleterious translocations that may be involved in the initiation of BZ-induced leukemia.

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